



Sex-related responses of European aspen (*Populus tremula* L.) to combined stress: TiO₂ nanoparticles, elevated temperature and CO₂ concentration



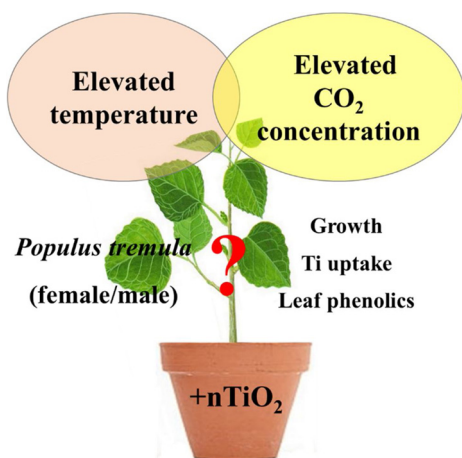
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GRAPHICAL ABSTRACT



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ABSTRACT

The combined effects of climate change and chemical contaminants on plant performance are still not well understood. Especially, whether different sexes of dioecious plants respond differently to combined stresses is unknown. In order to study the sex-related responses of European aspen to soil nTiO₂ contamination (0, 50, 300 mg kg⁻¹) under elevated temperature (+1.6 °C) and CO₂ (730 ppm), we conducted a study in greenhouses. Ti accumulated in roots exposed to nTiO₂ (1.1–3.3 and 2.7–21.1 mg kg⁻¹ in 50 and 300 mg kg⁻¹ treatments, respectively). Elevated CO₂ had no effects on Ti uptake, while elevated temperature increased it in the 300 mg kg⁻¹ treatment. Males grew taller than females under ambient conditions, but females had greater height and biomass increment under elevated temperature. In all climate treatments, nTiO₂ increased leaf phenolics in females by 12–19% and 15–26% at 50 and 300 mg kg⁻¹, respectively. Leaf phenolics decreased under elevated temperature, but increased under elevated CO₂ in both sexes. Results suggest that females have better chemical defense against nTiO₂ than males under future climate conditions. In the longer run, this may cause changes in the competitive abilities of both sexes, which again may affect sex ratios and genetic variation in nature.

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1. Introduction

Since industrialization, the increased human activities caused by rapid population growth and economic development have resulted in several environmental changes including elevated air temperature and CO₂ concentrations. According to most climate scenarios, global average temperature will increase by 1.5–4.8 °C and CO₂ concentrations are predicted to rise to 430–1000 ppm by the year 2100 compared to pre-industrial levels [1]. Elevated temperature and CO₂ affect the growth, productivity, physiology, and biochemistry of plants. In addition, the pH of the soil rhizosphere and the diversity and activity of rhizosphere microorganisms may be altered, which would concomitantly affect plant growth [2–5]. Plant secondary metabolites, such as phenolics, are not all necessary for growth, but may play a vital role in plant adaptability and defense [6]. Previous studies have shown that the concentrations of phenolic compounds in plants decreased under elevated temperature, and increased under elevated CO₂ concentration. One has also revealed significant interactions between elevated temperature and elevated CO₂, as elevated temperature diminished the positive effects of elevated CO₂ on plant phenolics [7–9]. However, the effects of elevated temperature and CO₂ can vary, depending on the exposure duration, compound, and plant species tested [8,10].

TiO₂ nanoparticles (nTiO₂) are extensively used in industrial and commercial products (e.g., cements, asphalts, paints, sunscreens, cosmetics, and coating) because of their photocatalytic properties [11,12]. Inevitably, the widespread use of nTiO₂ will lead to its continuous release into the environment, which makes it a potential environmental contaminant. Nano-TiO₂ is insoluble in soil and the nanoparticulate form is also the main chemical form that can be accumulated in plant tissues, especially in roots [13]. According to previous studies, nTiO₂ reduced or did not affect plant biomass, modified antioxidative enzyme activities and biochemical compositions in plants, and altered soil enzyme activities and bacterial communities [14–17]. One could expect that nTiO₂ could possibly interact with climate change factors, but this has seldom been tested. Du et al. [12] found that under elevated CO₂ concentration, nTiO₂ significantly reduced rice biomass and grain yield, and also changed the functional composition of soil microbial communities. Apart from this, little is known about the effects of nTiO₂ on plant performance under elevated temperature and CO₂ concentration.

European aspen (*Populus tremula* L.), a dioecious tree species, has great economic (e.g., a source for paper and construction industry) and ecological (e.g., host for herbivores and epiphytes) significance. It is widely distributed throughout Eurasian boreal and temperate ecosystems, and the populations of *P. tremula* are male dominated in most of the distribution areas [18]. In general, females invest more resources in reproduction and accumulation of secondary metabolites for defense than growth, while males invest more in growth and have higher herbivore abundance and damage [19–23]. Effects of elevated temperature and CO₂ on sex differences in plant growth may vary with plant species. For example, the positive effects of elevated temperature on growth were greater in males of *Salix myrsinifolia* Salisb. [22], while females had higher height and diameter growth than males of *Populus cathayana* Rehd. under elevated temperature [24]. Likewise, males of *P. cathayana* possessed greater increases in biomass under elevated CO₂ [25], but no sex differences in *S. myrsinifolia* were affected by CO₂ [26]. In addition, sex differences in defensive phenolics response to enhanced temperature and CO₂ are also variable, depending on different plant tissues, growing stage, and compound in question [22,26]. As there are different trade-offs between growth and defense between the sexes, plants may also have varying sex-related responses in growth and phenolics to soil nTiO₂ contamination under climate change. However, no studies have investigated the different responses of plant growth and phenolics to nTiO₂ in combination with elevated temperature and CO₂ between the sexes.

In order to investigate the sex-related effects of nTiO₂ in combination with elevated temperature and CO₂ on *P. tremula* seedlings, we

conducted a study in climate-regulated greenhouses. We analyzed the changes in plant height and diameter growth, biomass, leaf area, Ti uptake and leaf phenolics in females and males of *P. tremula* seedlings under single and combined effects of soil nTiO₂ contamination and enhanced temperature and CO₂ concentration. Our aims of this study were to answer the following questions. (1) As a relatively stable contaminant in soil, will nTiO₂ affect plant growth and leaf defensive phenolics, and will there be interactions between nTiO₂ and climate factors? (2) Whether plant tissues will accumulate Ti from nTiO₂ treatments, and how will elevated temperature and CO₂ affect the Ti uptake in plants? (3) How will these responses differ between female and male seedlings of *P. tremula*?

2. Materials and methods

2.1. Experimental setup

The study was conducted at Mekrijärvi Research Station (62°47'N, 30°58'E, University of Eastern Finland), for 11 weeks, from May 20 to August 5, 2015. Twelve greenhouses (16 m²) were randomly assigned to four combinations of two-level temperature and CO₂ concentration treatments (n = 3): ambient temperature + ambient CO₂ (C), elevated temperature + ambient CO₂ (T), ambient temperature + elevated CO₂ (CO₂), and elevated temperature + elevated CO₂ (T + CO₂). Ambient temperature (15.7 °C on average) and CO₂ concentration (400 ppm on average) were achieved by following the outside air temperature and CO₂ concentration through a modulated system. Temperature in the elevated treatment greenhouses was on average 1.6 °C higher than the ambient level, and CO₂ concentration was on average 730 ppm. The relative humidity was maintained at 60% in all the greenhouses. More technical details are described in Zhou et al. [27].

2.2. Soil preparation and plant materials

The main characteristics of the peat soil (Kekkilä Oy, Vantaa, Finland) used in this experiment were as follows: pH, 6.2; available nitrogen (N), 0.7 g kg⁻¹; available phosphorus (P), 0.4 g kg⁻¹; available potassium (K), 1.5 g kg⁻¹; total Ti, 24.5 mg kg⁻¹. The nTiO₂ (purity > 97%, particle size < 100 nm) was purchased from Sigma-Aldrich Co., St. Louis, MO, USA. These nanoparticles had a wide size distribution (diameter 20–100 nm) and the primary size was 20 ± 5 nm [14]. Specified amounts of nTiO₂ were added and mixed thoroughly into the soil to have concentrations of 0 (control), 50 (nTiO₂-50), and 300 (nTiO₂-300) mg kg⁻¹, based on previous studies [14,17]. The contaminated soils were incubated for two weeks before planting.

The *P. tremula* seedlings (five females and six males) used in this study were originally micropropagated from the dormant axillary buds of adult European aspen trees from different locations in eastern and southern Finland (Table S1). The mother trees were selected from distant locations to make sure that they belonged to different genotypes. The plantlets were regenerated *in vitro* on woody plant medium (WPM) supplemented with agar (8.5 g l⁻¹) and indole butyric acid (5 mg l⁻¹) at 23 ± 0.1 °C under a 16/8 h light/dark cycle supplied by plant growth lamps (GRO-LUX F36W, Havelis Sylvania, Erlangen, Germany) at a photosynthetically active radiation of ~70 μmol m⁻² s⁻¹ for root development. The plantlets were acclimated in a greenhouse, where the environmental conditions were maintained at temperature of 20 ± 3 °C, air relative humidity of 70%, and photoperiod of 16/8 h light/dark. They were transferred to Mekrijärvi Research Station and then planted in plastic pots (4 l in volume) on May 20, 2015. All pots were watered regularly and fertilized twice (on June 4 and July 6) with Kekkilä Peat Superex (N: P: K = 12: 5: 27, %, Kekkilä Oy, Vantaa, Finland). There were 396 plants in total in this study (3 individual seedlings of each genotype per greenhouse × 11 genotypes × 12 greenhouses).

2.3. Growth measurements and sampling

The height growth and diameter growth were measured every second week (1, 15, 29, 43, 57, and 71 days after planting) during the experiment. In this experiment, 8 of the 11 genotypes of the control individuals (without nTiO₂ contamination) used were the same as Sobuj et al. [28] for the height, diameter, and biomass measurements. In Sobuj et al. [28], four female and four male genotypes of *P. tremula* (four replications) were grown in clean soils under elevated temperature and CO₂ concentration, stem phenolics were measured; in this study, five females and six males (three replications) were grown in both clean and nTiO₂ contaminated soils, and leaf phenolics were measured. At the end of the experiment, two mature leaves from each individual were collected for leaf area measurements and leaf phenolic analyses. The remaining aboveground parts of all seedlings were harvested for biomass measurements. All samples were dried at room temperature, and then separated into leaves and stems, and weighed. Leaf area (LA, cm²) was measured with a portable leaf area meter LI-3000C (LI-COR, Lincoln, NE, USA), and specific leaf area (SLA, cm² g⁻¹) was calculated as the ratio of leaf area to leaf dry weight.

2.4. Analyses of Ti in leaves, stems, and roots

Dried plant samples (~0.5 g) were digested with HNO₃ and HClO₄ (4:1, v/v) and Ti concentrations were analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES, Optima 5300, PerkinElmer, Waltham, Mass., USA) according to Du et al. [12]. Three replications were performed for each sample. The same solution without leaf samples was used as control. A calibration check standard was prepared using a titanium stock solution GSBG62014-90 (Central Iron & Steel Research Institute, Beijing, China). This was analyzed as a sample to verify analyte concentration and instrument calibration.

2.5. Phenolic analyses and compound identification

Dried leaf samples (~7 mg) were extracted and analyzed by high-performance liquid chromatography (HPLC, 1100 series, Agilent, Santa Clara, CA, USA) according to Nybakken et al. [22]. The compounds were identified by mass spectrometry using a quadrupole time-of-flight mass spectrometer (QTOF/MS, 6540 series, Agilent, Santa Clara, CA, USA) with an ultra-HPLC with a diode array detector (UHPLC-DAD, 1200 series, Agilent, Santa Clara, CA, USA) (Table S2). Detailed instrument conditions are described in Randriamanana et al. [29]. The reference mass *m/z* 922.0098 was used for accurate mass measurements. The mass accuracy or error term (ppm) was calculated as follows: (measured mass – calculated mass) × 10⁶/calculated mass. Quantification of flavonoids and phenolic acids was done at 320 nm and salicylates at 270 nm based on the standards as follows: salicin (Sigma-Aldrich Chemie, Steinheim, Germany) for diglucoside of salicyl alcohol and salicin; chlorogenic acid (Sigma-Aldrich Chemie, Steinheim, Germany) for chlorogenic acid, neochlorogenic acid, and *p*-OH-cinnamoyl quinic acid; salicortin (Sigma-Aldrich Chemie, Steinheim, Germany) for salicortin and disalicortin; hyperoside (Roth, Karlsruhe, Germany) for quercetin 3-arabinoglucoside and quercetin 3-glucuronide; astragalol (Extrasynthese, Genay Cedex, France) for kaempferol 3-arabinoglucoside and kaempferol 3-glucuronide; tremulacin (Apin Chemicals, Abingdon, UK) for tremulacin; *p*-OH-cinnamic acid (Sigma-Aldrich Chemie, Steinheim, Germany) for *p*-OH-cinnamic acid derivatives, cinnamoyl salicortin, and *p*-OH-cinnamoyl salicortin; kaempferol 3-rhamnoside (Sigma-Aldrich Chemie, Steinheim, Germany) for mono-coumaroyl-kaempferol 3-rhamnoside.

2.6. Statistical analyses

All data were analyzed by a linear mixed model using SPSS 22 (IBM, Armonk, NY, USA). Temperature, CO₂, nTiO₂, and sex were set as fixed

factors, and genotype and greenhouse as random factors. Pairwise comparisons were made between nTiO₂ treatments using the least significant difference test. When more than one measurement was made per individual from the same treatment (e.g., leaf area), the average was used in analyses. For analyzing height and diameter, the increment (end height/diameter – start height/diameter) was used, and start height and start diameter were used as covariates. The data were ln(*x*)- or sqrt(*x*)-transformed when necessary (see Table S3 and S4). Nonparametric tests were used when data did not meet the requirements of parametric tests. In addition, a graphic vector analysis (GVA) was performed to further study the effects of elevated temperature, elevated CO₂, nTiO₂, and sex on leaf phenolics and biomass accumulation. In the GVA, the relative values of content (*x*), concentration (*y*) of each phenolic group and leaf biomass (*z*) are plotted three-dimensionally onto a diagram. The mean values in control soil and control greenhouses were used as the reference points (*x* = *y* = *z* = 1). The effects of the treatments are expressed by the magnitude and direction of vectors. The directional changes indicate possible concentration, dilution, and excess or reduced synthesis. More details of the construction and interpretation of GVA are described in Haase et al. [30] and Koricheva [31].

3. Results

3.1. Growth

Nano-TiO₂ alone showed no main effects or interactions with other factors on height growth, diameter growth or biomass of *P. tremula* seedlings (Figs. 1 and 2, Table S3). Height growth, diameter growth and biomass significantly increased under elevated temperature, while only the height increment was reduced under elevated CO₂ compared to ambient treatments (Fig. 1, Fig. 2, Table S3) (based on Sobuj et al. [28], we had 3 more genotypes of those control individuals for height, diameter and biomass). Males showed faster height growth than females under ambient conditions, but the height increment was greater in females than in males under elevated temperature (significant T × sex interaction) (Fig. 1a, Table S3). Although no significant differences in biomass were detected under ambient conditions, there were T × sex interactions on leaf, stem and total shoot biomass (Table S3), as the increments in biomass were greater in females than in males under elevated temperature.

Leaf area (LA) and specific leaf area (SLA) were unaffected by nTiO₂ (Table 1). The main effect of elevated temperature on LA was not significant while LA tended to be smaller than controls under elevated CO₂ (Table 1). SLA significantly increased by 19% and 9% for females and males, respectively, under elevated temperature, but decreased by 15% and 19% for females and males, respectively, under elevated CO₂ compared to ambient treatments (Table 1). In addition, elevated temperature eliminated the negative effects of elevated CO₂ on SLA (significant T × CO₂ interaction) (Table 1). Males had greater LA and SLA than did females (Table 1).

3.2. Ti uptake in plant tissues

Ti was not detected in leaves or stems in any soil treatments, neither in roots in control soils (Table 2). However, it was significantly accumulated in roots in nTiO₂ contaminated soils (Table 2). The concentration of Ti in roots was much higher in nTiO₂-300 soils than in nTiO₂-50 soils (*p* < 0.001), and it was unaffected by elevated CO₂ (*p* = 0.463) (Table 2). Under elevated temperature, there was an increase in Ti concentration in roots (*p* = 0.007), but only in nTiO₂-300 soils (significant T × nTiO₂ interaction, *p* = 0.014) (Table 2). No significant differences in root Ti concentration were detected between the sexes (*p* = 0.442).

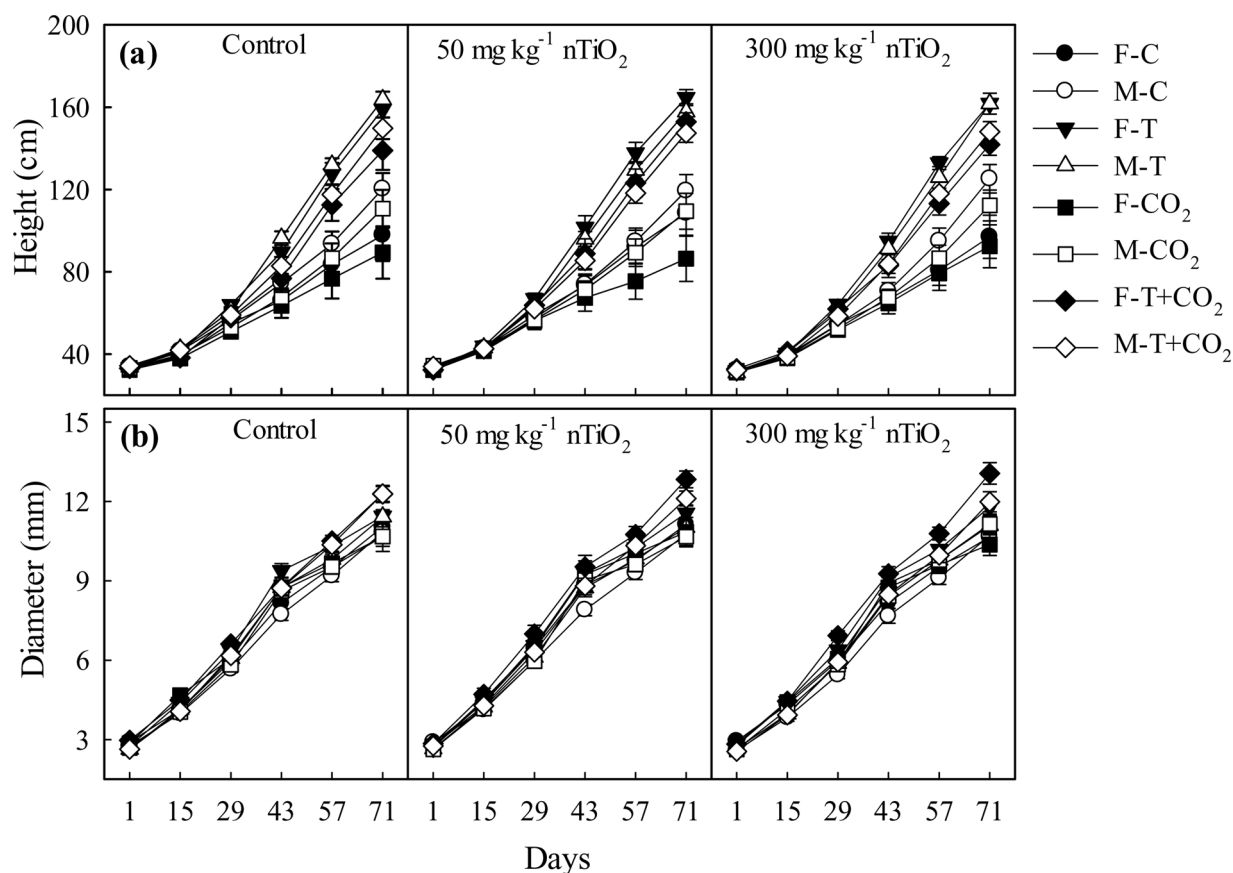


Fig. 1. Mean (\pm SE) height (a) and diameter (b) growth of females (F) and males (M) of *P. tremula* seedlings under control (C), elevated temperature (T), elevated CO₂ (CO₂), and T + CO₂ without and with nTiO₂ in soil.

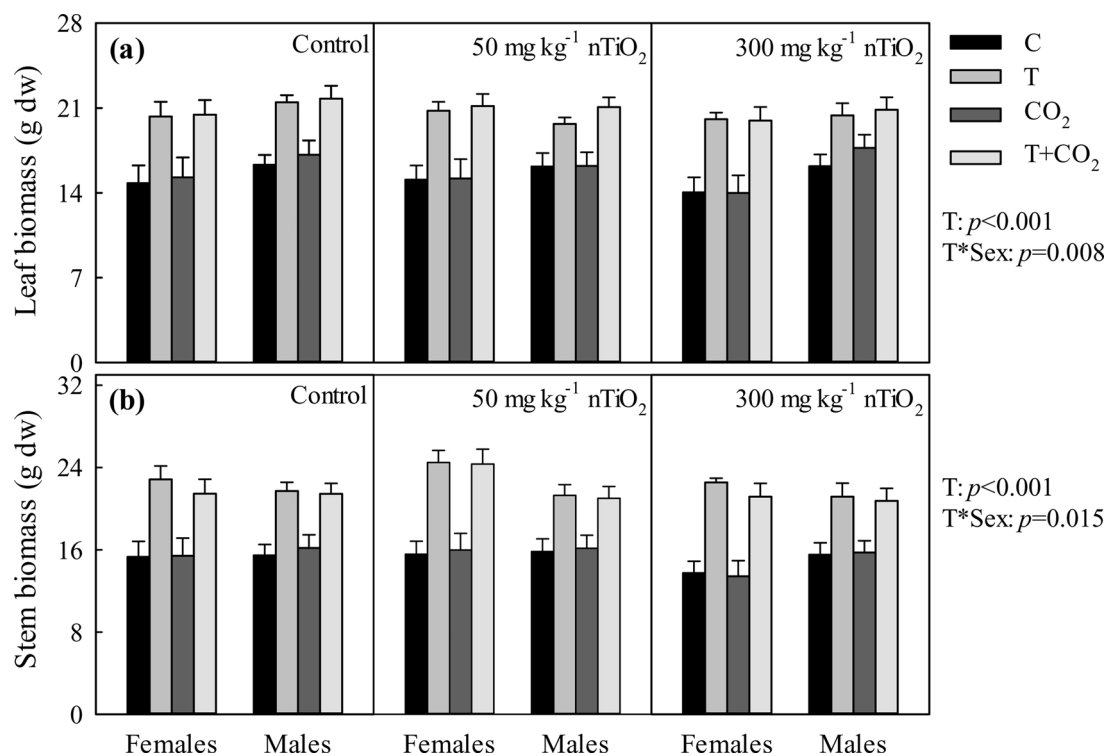


Fig. 2. Mean (\pm SE) leaf (a) and stem (b) biomass of females and males of *P. tremula* seedlings under control (C), elevated temperature (T), elevated CO₂ (CO₂), and T + CO₂ without and with nTiO₂ in soil. Statistically significant results ($p < 0.05$) from the linear mixed model are presented.

Table 1

Mean (\pm SE) leaf area (LA) and specific leaf area (SLA) of females and males of *P. tremula* seedlings under control (C), elevated temperature (T), elevated CO₂ (CO₂), and T + CO₂ with different nTiO₂ concentrations in soil. *F*-values and levels of significance (*p*) from the linear mixed model for T, CO₂, nTiO₂, Sex, and their interactions are presented. Statistically significant results are marked with bold (*p* < 0.05).

		LA (cm ²)			SLA (cm ² g ⁻¹)		
nTiO ₂ (mg kg ⁻¹)		0	50	300	0	50	300
C	Females	139.6 \pm 13.8	134.8 \pm 9.3	150.1 \pm 6.8	340.8 \pm 6.4	338.3 \pm 6.7	365.9 \pm 3.4
	Males	163.9 \pm 8.1	161.5 \pm 6.0	170.5 \pm 4.7	388.9 \pm 15.2	387.0 \pm 12.4	392.3 \pm 9.7
T	Females	143.1 \pm 10.5	148.0 \pm 4.2	147.7 \pm 3.6	412.9 \pm 13.7	434.3 \pm 24.3	404.3 \pm 11.7
	Males	172.4 \pm 8.5	161.6 \pm 6.0	169.9 \pm 4.5	418.9 \pm 9.8	427.2 \pm 17.1	425.0 \pm 11.3
CO ₂	Females	130.3 \pm 18.0	119.9 \pm 10.0	120.6 \pm 7.5	302.1 \pm 4.0	299.1 \pm 4.9	287.5 \pm 7.1
	Males	146.7 \pm 6.9	126.8 \pm 6.0	152.8 \pm 4.9	326.9 \pm 7.9	317.4 \pm 14.0	307.9 \pm 7.6
T + CO ₂	Females	142.8 \pm 10.1	131.6 \pm 6.4	124.4 \pm 4.0	389.3 \pm 16.2	373.2 \pm 17.9	361.7 \pm 10.5
	Males	148.8 \pm 6.1	142.0 \pm 4.1	146.5 \pm 6.9	363.3 \pm 3.5	375.5 \pm 8.6	413.1 \pm 10.9
<i>F_T</i> (<i>p</i>)		0.402 (0.544)			75.775 (0.000)		
<i>F_{CO2}</i> (<i>p</i>)		3.842 (0.085)			52.045 (0.000)		
<i>F_{nTiO2}</i> (<i>p</i>)		1.369 (0.256)			0.133 (0.876)		
<i>F_{Sex}</i> (<i>p</i>)		22.378 (0.000)			8.384 (0.004)		
<i>F_{T × CO2}</i> (<i>p</i>)		0.099 (0.760)			6.143 (0.038)		
<i>F_{T × nTiO2}</i> (<i>p</i>)		0.964 (0.382)			0.229 (0.795)		
<i>F_{T × Sex}</i> (<i>p</i>)		0.527 (0.468)			2.316 (0.129)		
<i>F_{CO2 × nTiO2}</i> (<i>p</i>)		0.632 (0.532)			0.483 (0.618)		
<i>F_{CO2 × Sex}</i> (<i>p</i>)		0.496 (0.482)			0.673 (0.413)		
<i>F_{nTiO2 × Sex}</i> (<i>p</i>)		0.474 (0.623)			0.902 (0.407)		
<i>F_{T × CO2 × nTiO2}</i> (<i>p</i>)		0.226 (0.798)			1.836 (0.161)		
<i>F_{T × CO2 × Sex}</i> (<i>p</i>)		0.021 (0.886)			0.417 (0.519)		
<i>F_{T × nTiO2 × Sex}</i> (<i>p</i>)		0.007 (0.993)			1.609 (0.202)		
<i>F_{CO2 × nTiO2 × Sex}</i> (<i>p</i>)		0.680 (0.507)			0.727 (0.484)		
<i>F_{T × CO2 × nTiO2 × Sex}</i> (<i>p</i>)		0.451 (0.638)			0.506 (0.603)		

3.3. Leaf phenolics

Three groups of phenolics were detected in *P. tremula* leaves, including salicylates (76–82% of leaf phenolics, all treatments), flavonoids (12–17%), and phenolic acids (6–8%) (Fig. 3). Overall, the concentration of leaf total phenolics decreased by 6–9% under elevated temperature, while it increased by 2–7% under elevated CO₂ in comparison with ambient in all soil treatments (Fig. 3d, Table S4). It was also increased by 12–19% (nTiO₂-50) and 15–26% (nTiO₂-300) when nTiO₂ was present in soils in all climate treatments, but only in females (significant nTiO₂ × sex interaction) (Fig. 3d, Table S4). No significant differences in total phenolics were detected between the two nTiO₂ concentrations (*p* = 0.121).

The responses of total salicylates under elevated temperature, elevated CO₂, and nTiO₂ were similar to that of leaf total phenolics (Fig. 3a,d, Table S4). In total, 7 salicylates were detected, and salicortin (49–62%) and tremulacin (23–41%) were the most abundant. The concentration of salicortin decreased under elevated temperature and increased under elevated CO₂, while that of tremulacin increased under elevated temperature (Table S4). There were nTiO₂ × sex interactive effect on salicortin and tremulacin (Table S4), as the increases caused

by nTiO₂ were only found in females.

Six flavonoid compounds were identified, of which quercetin 3-glucuronide (45–59%), kaempferol 3-glucuronide (15–27%), and quercetin-arabinofuranoside-glucoside (11–18%) were the most concentrated. The concentration of total flavonoids was higher in females than in males (Fig. 3b, Table S4). In female seedlings, the concentration of total flavonoids decreased under elevated CO₂ and increased when nTiO₂ was present in soils (Fig. 3b, Table S4), which reflects the effects on quercetin 3-glucuronide and quercetin-arabinofuranoside-glucoside (Table S4). Females had higher levels of kaempferol 3-glucuronide than males (Table S4). The concentration of kaempferol 3-glucuronide decreased in females, but increased in males under elevated CO₂ in control soils (significant CO₂ × sex interaction). Nano-TiO₂, on the other hand, increased it only in females in all climate treatments (significant nTiO₂ × sex interaction) (Table S4).

Neochlorogenic acid (47–57%), chlorogenic acid (17–33%), and *p*-OH-cinnamoyl quinic acid (12–22%) were the most abundant phenolic acids. Females had higher neochlorogenic acid and chlorogenic acid levels, but males had higher concentrations of *p*-OH-cinnamoyl quinic acid (Table S4). In all soil treatments, the concentration of chlorogenic acid decreased under elevated temperature, but increased under

Table 2

Mean (\pm SE) Ti concentration in females and males of *P. tremula* seedlings under control (C), elevated temperature (T), elevated CO₂ (CO₂), and T + CO₂ with different nTiO₂ concentrations in soil.

Ti (mg kg ⁻¹)		leaves			stems			roots		
nTiO ₂ (mg kg ⁻¹)		0	50	300	0	50	300	0	50	300
C	Females	nd			nd			nd	1.7 \pm 0.5 a	2.9 \pm 0.4 b
	Males								1.7 \pm 0.6 a	2.7 \pm 0.6 b
T	Females								1.7 \pm 0.3 a	17.9 \pm 5.4 c
	Males								1.9 \pm 0.4 a	13.9 \pm 3.1 c
CO ₂	Females								1.1 \pm 0.9 a	4.4 \pm 0.7 b
	Males								1.7 \pm 0.4 a	4.2 \pm 1.2 b
T + CO ₂	Females								3.3 \pm 1.8 ab	21.1 \pm 5.2 c
	Males								2.5 \pm 1.3 ab	19.4 \pm 4.0 c

nd: below detection limit.

Different letters indicate statistically significant differences at *p* < 0.05.

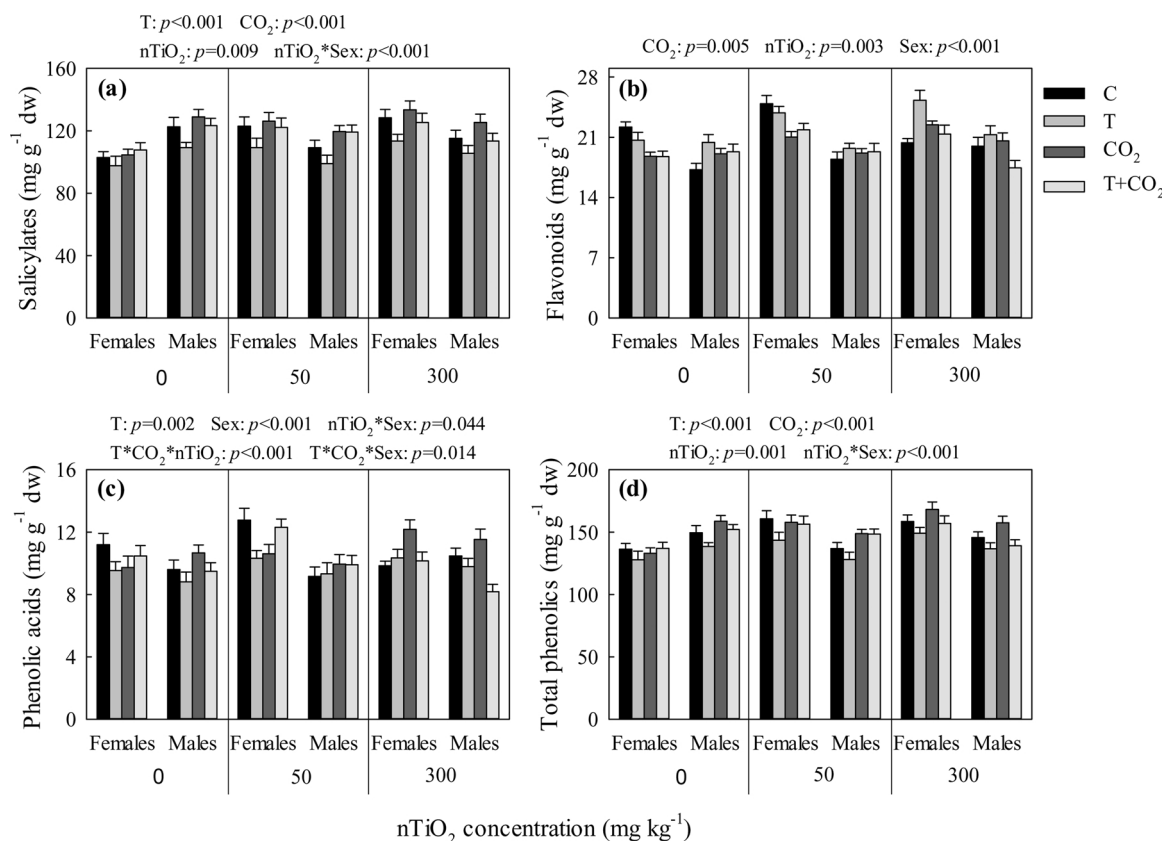


Fig. 3. Mean (\pm SE) concentrations of leaf total salicylates (a), flavonoids (b), phenolic acids (c) and total phenolics (d) in different sexes of *P. tremula* seedlings under control (C), elevated temperature (T), elevated CO₂ (CO₂), and T + CO₂ without and with nTiO₂ in soil. Statistically significant results ($p < 0.05$) from the linear mixed model are presented.

elevated CO₂ (Table S4). The concentration of *p*-OH-cinnamoyl quinic acid decreased under elevated temperature, but only in males (significant T \times sex interaction) (Table S4). Nano-TiO₂ at 50 mg kg⁻¹ increased the concentration of total phenolic acids by 8–17% in all climate treatments, but only in females (significant nTiO₂ \times sex interaction), while nTiO₂ at 300 mg kg⁻¹ decreased it under T + CO₂ when compared to control soils (significant T \times CO₂ \times nTiO₂ interaction) (Fig. 3c, Table S4). In control soils, total phenolic acids were higher in males under elevated CO₂, but they were higher in females under T + CO₂ (significant T \times CO₂ \times sex interaction) (Fig. 3c, Table S4).

To further study the changes in phenolic allocation in response to elevated temperature, elevated CO₂, and nTiO₂ relative to control plants, GVA diagrams were made for the three phenolic groups (Figs. 4 and 5). In control soils, elevated temperature induced a dilution effect of increased leaf biomass on leaf salicylates, flavonoids, and phenolic acids in females, as well as salicylates and phenolic acids in males, but the synthesis of flavonoids in males was increased under elevated temperature (Fig. 4a–f). Elevated CO₂ and T + CO₂ increased the accumulation of all three phenolic groups in males while only salicylates in females (Fig. 4a, b, d, f). Additionally, elevated CO₂ reduced the synthesis of flavonoids and phenolic acids but T + CO₂ resulted in a dilution effect on these two phenolic groups in females (Fig. 4c, e). In all treatments, both nTiO₂ levels increased the synthesis of salicylates in females (Fig. 5a). However, nTiO₂ decreased the synthesis of salicylates in males under ambient conditions (Fig. 5b). Flavonoid accumulation in both sexes was increased by nTiO₂ under elevated temperature and T + CO₂ (Fig. 5c, d). The synthesis of phenolic acids was increased by nTiO₂-50 but reduced by nTiO₂-300 in females under ambient conditions, while males showed the opposite (Fig. 5e, f).

4. Discussion

4.1. Growth

All the measured growth parameters were unaffected by nTiO₂, which is in accordance with earlier studies on vegetables (*Lycopersicon esculentum* Mill.), crops (*Triticum aestivum* L. and *Phaseolus vulgaris* L.), and some aquatic plants (*Rumex crispus* L. and *Elodea Canadensis* Michx.) [15,32]. Nano-TiO₂ has been reported to be less toxic than other metal-based nanoparticles (e.g., Ag and ZnO nanoparticles) because of its insolubility in soils [13,33]. However, the effects of nTiO₂ on plant growth have also been positive or negative, depending on different concentrations and particle size of nTiO₂, experimental setups and systems [32,34].

Plant height, diameter, biomass, and SLA increased under elevated temperature, which agrees with the findings of Randriamanana et al. [23]. However, unlike most earlier studies [25,26,35], we found that height and SLA decreased under elevated CO₂, and diameter and biomass were not affected. Similar results from studies on *Picea abies* (L.) Karst. were noted by Sallas et al. [36]. Tjoelker et al. [37] investigated the growth responses of five boreal tree species to elevated CO₂ at different temperatures and found that effects of elevated CO₂ on growth were minimal or even suppressed at low temperatures, but maximal towards optimal growth temperatures. The average temperatures in our experiment (15.7 °C and 17.3 °C under ambient and elevated temperature, respectively) might be considerably below the optimum growth temperature of *P. tremula* seedlings. Decreased SLA under elevated CO₂, and increased biomass and SLA under elevated temperature might be associated with greater transpiration stress, reflecting the demand for increased water use efficiency [36]. Elevated CO₂ may increase photosynthesis and reduce stomatal conductance [38,39], and as elevated

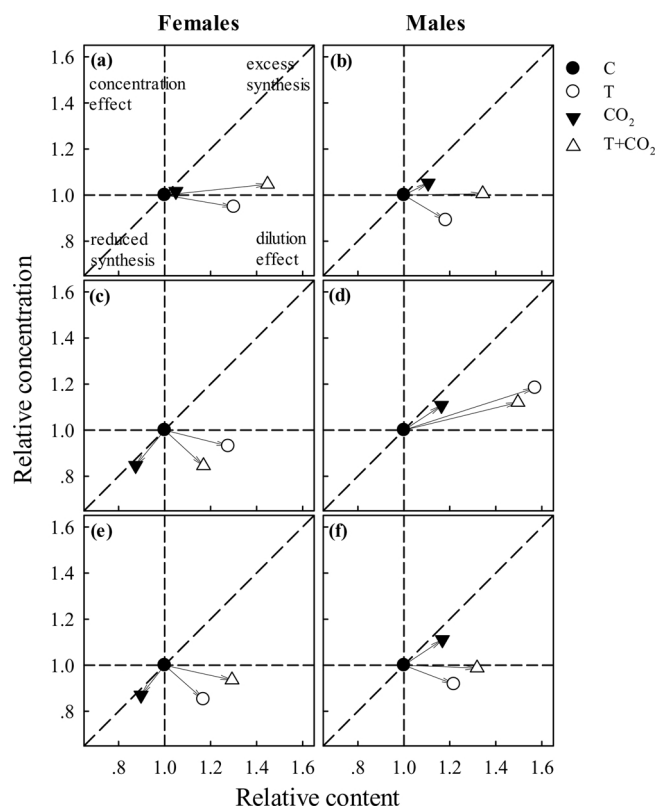


Fig. 4. Graphic vector diagrams of salicylate (a, b), flavonoid (c, d), and phenolic acid (e, f) in leaves of females and males of *P. tremula* seedlings under control (C), elevated temperature (T), elevated CO₂ (CO₂), and T + CO₂ without nTiO₂ in soil. The oblique dashed lines represent reference lines for no change in leaf biomass; the vertical dashed lines represent the reference lines for no change in phenolic content; and the horizontal dashed lines represent the reference lines for no change in phenolic concentration. The direction of the vector arrows indicates the trend of the treatments' effects (concentration, dilution, and excess or reduced synthesis); the length of the vector arrows indicates the magnitude of the effects.

temperature increases photosynthesis more than transpiration within a certain temperature range (under the optimal temperature) [40], the water use efficiency might have increased. Although not measured here, previous studies have reported an increase of the water use efficiency in leaves of *P. tremula* under elevated temperature [23] and in leaves of *Oryza sativa* L. under elevated CO₂ concentration [10].

4.2. Ti uptake in plant tissues

Although the nanoparticulate form is the main chemical form that accumulate in plants, the component metal (Ti) can also be accumulated by plant tissues from nTiO₂ treatments, especially in roots [13,32]. Plant roots are more sensitive to the soil contaminants as they are directly exposed to the contaminants in soils [41]. In this study, Ti was detected only in roots in nTiO₂ contaminated soils. Elevated CO₂ showed no effects on root Ti uptake, which is in agreement with the results of Du et al. [12]. However, Ti uptake in roots increased significantly under elevated temperature. Elevated temperature may increase the number of lateral roots or production of fine roots within a certain temperature range [42,43]. Fine roots are thought to be the main site of element absorption due to their high surface area to mass ratio [43]. In our experiment, we observed that seedlings had more fine roots under elevated temperature (Fig. S1), which might have resulted in more absorption of Ti from nTiO₂ contaminated soils. However, the concentration of Ti in roots was not high enough to affect taproot and plant shoot growth in our study.

4.3. Leaf phenolics

The concentration of leaf total phenolics decreased under elevated temperature and increased under elevated CO₂, which supports previous findings [9,23]. Generally, nTiO₂, as a soil contaminant, stimulated the accumulation of total phenolics in *P. tremula* leaves, but no interactions were found between nTiO₂ and the climatic factors. Environmental stressors can affect plant phenolic concentrations by diverting substrates from primary processes of growth into secondary metabolism, which may result in trade-offs between growth and defense [e.g. [44]. Here, we found that plant growth significantly increased but leaf total phenolics decreased under elevated temperature, while elevated CO₂ and nTiO₂ did not affect growth but increased total phenolics. This can be explained according to Bryant et al. [45] and Herms et al. [46], as plants allocate more resources to growth than secondary metabolites under elevated temperature, while the carbon surpluses are high enough to satisfy or to prioritize the production of phenolics over growth under elevated CO₂ and in the presence of nTiO₂.

Salicylates are the major phenolics and have important functions in *Populus* species, as they can protect plants against generalist herbivores, pathogens and abiotic stresses [29,47,48]. Flavonoids and phenolic acids are proved to have good antioxidant properties, as they can delay or inhibit the oxidation by scavenging radical species (e.g., ROS) [49]. In our study, *P. tremula* seedlings accumulated much more salicylates than flavonoids and phenolic acids, suggesting that *P. tremula* prioritize the accumulation of salicylates in leaves. Total salicylates and flavonoids increased when nTiO₂ was present in soils. Nano-TiO₂ has been reported to alter the soil bacterial community composition under either ambient or elevated CO₂ conditions, which may influence the composition and quality of plant root exudates [12,17]. This can further affect plant absorption, utilization, and production of nutrient under climate change [12], and thus change the production of phenolics. The concentration of total salicylates and phenolic acids significantly decreased, but that of several individual phenolic compounds increased under elevated temperature. Total salicylates and several individual phenolic acids increased, but total flavonoids decreased under elevated CO₂. These variable responses show that plants can acclimate or adapt to multiple environmental stresses by regulating the production of phenolics.

4.4. Sex differences

The sexes of dioecious plants may allocate resources differently, and often females invest more resources in chemical defense and reproduction while males invest more in growth [19–21]. These sexual differences can be influenced by abiotic stresses [22,23]. In our study, females had higher concentrations of total flavonoids and phenolic acids than did males. Moreover, nTiO₂ induced an increase in concentrations of all three phenolic groups and total phenolics in females, but not in males. These results suggest that females have a stronger chemical response to nTiO₂. This may indicate that females are better protected from soil nTiO₂ contamination than males and males may get more herbivore damages in nTiO₂ contaminated soils. Although no interactions between climatic factors and sex were detected in leaf total phenolics, there were T × sex, CO₂ × sex, and T × CO₂ × sex interactions in several individual phenolics, indicating that the sex differences in defensive phenolics response to elevated temperature and CO₂ can vary depending on different compounds.

The sex differences in phenolics response to soil nTiO₂ contamination in combination with elevated temperature and CO₂ may alter plant competitive abilities in different sexes under future environmental conditions. Female plants may be more sensitive to nTiO₂ than males, as nTiO₂ stimulated more accumulation of leaf phenolics in females.

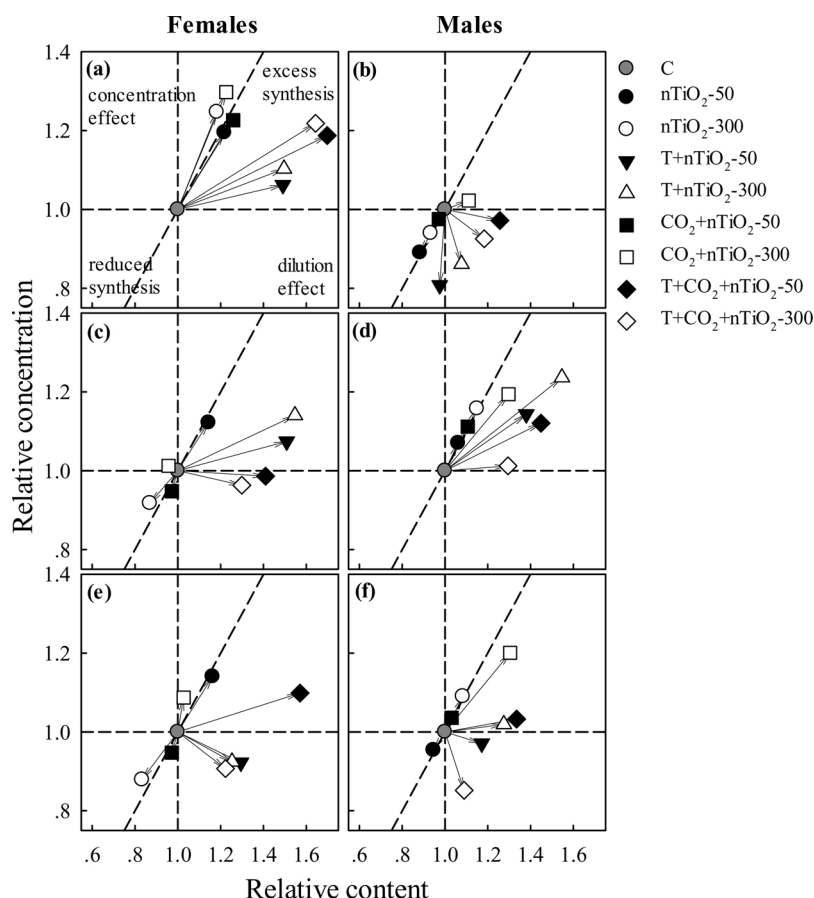


Fig. 5. Graphic vector diagrams of salicylate (a, b), flavonoid (c, d), and phenolic acid (e, f) in leaves of females and males of *P. tremula* seedlings under control, elevated temperature (T), elevated CO_2 (CO_2), and T + CO_2 with different nTiO₂ concentrations in soil. The mean value in control soil and control greenhouse was used as the reference point (C). The oblique dashed lines represent reference lines for no change in leaf biomass; the vertical dashed lines represent the reference lines for no change in phenolic content; and the horizontal dashed lines represent the reference lines for no change in phenolic concentration. The direction of the vector arrows indicates the trend of the treatments' effects (concentration, dilution, and excess or reduced synthesis); the length of the vector arrows indicates the magnitude of the effects.

5. Conclusions

The changes in plant growth and in the quantity and quality of defensive phenolics under changed climate conditions and soil nTiO₂ contamination will affect the environmental adaptability of different sexes of *P. tremula*. We assume that in the long run, the competitive abilities of different sexes of *P. tremula* based on nTiO₂ induced defense will be changed. Females will have better chemical protection and defense against nTiO₂, while males may be more susceptible to herbivores, and the sex ratio and genetic diversity of *P. tremula* may be changed. That may have negative effects on the aspen sexual reproduction and further species evolution.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jhazmat.2018.03.031>.

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